T_m filtering by ^1H-methyl labeling in a deuterated protein for pulsed double electron–electron resonance EPR†

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Modulating the phase-memory relaxation time (T_m) of a spin label by introducing ^1H-methyl groups in a perdeuterated protein background is used in DEER experiments to assign interactions in multimodal P(r) distributions. Proof of principle is demonstrated using Protein A where one nitroxide label occupies two distinct regions of conformational space. The presence of a single protonated methyl group in close proximity (4–8 Å) to only one of the two nitroxide rotamer ensembles results in a selective and substantial decrease in T_m, manifested by differential decay of the peak intensities in the bimodal P(r) distance distribution as a function of the total dipolar evolution time. This form of T_m filtering will facilitate DEER structural analysis of biomolecular systems with three spin labels, including complexes involving multimeric proteins.

Pulsed double electron–electron resonance (DEER) EPR spectroscopy provides a means of accurately measuring long-range distances between pairs of spin labels and, as such, is an invaluable tool for conformational analysis of proteins and other biological macromolecules.1,2 With complete deuteration of both protein and solvent, distances up to 170 Å can potentially be measured.3–5 When more than two spin labels are present, analysis can prove challenging, not only due to artefacts arising from multi-spin effects1,6,7 but because assignment of distances to specific interactions between paramagnetic centres may be difficult a priori. One approach to resolve this issue makes use of orthogonal spin-labeling to probe interactions between different spin labels (e.g., a nitroxide and a paramagnetic metal centre such as Gd3+ or Cu2+) separately from those between like spin labels.8–11 Here we propose another strategy that makes use of specific ^1H methyl labeling in a perdeuterated protein background,12 thereby permitting phase-memory relaxation time (T_m) filtering in DEER, analogous to T_2 filtering in solution NMR.13 We demonstrate this approach on a model system, Protein A, in which one of the nitroxide spin labels occupies two distinct regions of conformational space giving rise to two separate distances in the DEER-derived P(r) probability distance distribution.

The model system employed is AviTag-Protein A with the nitroxide spin label (R1) attached to engineered cysteine residues close to the N- (Q39C) and C- (K88C) termini of the ordered Protein A domain (residue numbering according to the complete construct which includes the AviTag; the Protein A domain extends from residues 30 to 90, and residues 1–38 are disordered).14 Previous Q-band DEER has shown the presence of two clearly resolved distances in the P(r) distribution, at 33 and 38 Å,14 arising from the Q39C-R1 label occupying two distinct regions of conformational space (labeled a and b in Fig. 1A), as judged by the predicted P(r) distribution generated from the atomic coordinates using the spin-label rotamer library program MMM.15,16

Hahn spin echo decay curves recorded with a two-pulse Hahn echo sequence (ESI,† Fig. S1A) with the nitroxide label either at the N- (Q39C-R1; Fig. 1A) or C- (K88C-R1; Fig. 1B) terminal ends of the ordered Protein A domain were recorded on four samples of AviTag-Protein A in fully deuterated solvent (70% D_2O/30% d_8-glycerol v/v); fully deuterated, fully protonated, and either Leu (C^13H^13 and C^15H^15) or Ile (C^13H^13) methylprotonated on a perdeuterated background (see ESI† for details of protein expression, purification, nitroxide labeling, deuteration, and incorporation of protonated Leu or Ile methyl groups). There are 7 leucines (Fig. 1A, right) and 2 isoleucines (Fig. 1B, right). The apparent T_m (T_m^app) values for the fully deuterated and fully protonated samples are ~35 and 9 µs, respectively (Fig. 1A and B). For Q39C-R1, the introduction of Leu ^1H-methyl groups in a perdeuterated background results in a two-fold reduction in the T_m^app value to ~17 µs (Fig. 1A); no reduction in T_m^app, however, is observed for the corresponding K88C-R1 sample (Fig. 1B). Introduction of C^13H^13 methylprotonated Ile in a perdeuterated background results in only a minimal reduction in the T_m^app value (to ~32 µs) for both the
Q39C-R1 and K88C-R1 samples (Fig. 1A and B), and it seems likely that this small reduction may in fact be due to the presence of a small amount of residual H$_2$O owing to incomplete exchange with the D$_2$O buffer. It has previously been shown that protons, and especially methyl groups in close proximity (4–8 Å) to the unpaired electron of a nitroxide spin label are the largest contributor to transverse electron relaxation, and examination of the Protein A structure (Fig. 1, right panels) shows that our results are fully consistent with this phenomenon, since only the Q39C-R1 spin label in conformer b is sufficiently close to a methyl group (specifically the two methyls of Leu35; Fig. 1A and also Fig. S2, ESI†) to induce a significant reduction in T$_{\text{app}}$.

Fig. 2 presents the results for Leu methyl-protonated, otherwise deuterated [Leu-CH$_3$/2H]-AviTag-Protein A spin-labeled at both Q39C-R1 and K88C-R1 (Fig. 2A). As expected, the T$_{\text{app}}^\text{m}$ for the Hahn spin-echo decay curve of the Q39C-R1/K88C-R1 doubly-spin labeled sample is intermediate between that for the Q39C-R1 and K88C-R1 single spin-labeled samples (Fig. 1B). DEER data were recorded with a refocussed four-pulse scheme (Fig. S1B, ESI†) varying the length of the second echo period T (= 2t$_2$), keeping the length of the acquisition time t$_{\text{max}}$ constant at 4 μs, with the exception of the data at t$_2$ = 4 μs, where t$_{\text{max}}$ was set to 3 μs (Fig. S3 and ESI† Methods). For t$_{\text{max}}$ = 3 and 4 μs, the upper limits for an accurate mean distance determination are 50(t$_{\text{max}}$/2)$^{1/3}$ = 57 and 63 Å, respectively; the upper limits for an accurate determination of the
width of the $P(r)$ distribution are $40(t_{max}/2)^{1/3} = 46$ and 50 Å, respectively.$^1$ The DEER echo curves were analysed to generate $P(r)$ distributions using five different methods: model free Tikhonov regularization with the program DeerAnalysis without (Fig. 2C) and with (Fig. S4A, ESI†) validation;$^{24}$ the program DD in which the DEER data are modelled as originating from the sum of two Gaussians (Fig. S5A, ESI†)$^{25}$ the program WavPDS (wavelets for pulse dipolar signals) to filter out noise using a wavelet denoising method,$^{26}$ followed by Tikhonov regularization$^{24}$ (Fig. S6A, ESI†); and WavPDS followed by singular value decomposition (SVD) (Fig. S7A, ESI†).$^{27,28}$ Since model-independent Tikhonov regularization, model-dependent Gaussian modelling and model-independent SVD represent three completely different approaches for extracting $P(r)$ distributions from DEER echo curves, a comparison of the results provides an independent means of assessing the accuracy of the resulting $P(r)$ distributions.$^{29,30}$ All five methods of analysis yield quantitatively very similar results, in which the integrated intensity of the $b$ component of the bimodal $P(r)$ distribution centred at $\sim 33$ Å decays more rapidly than that of the $a$ component centred at $\sim 38$ Å. Thus, one can immediately conclude that the $T_m$ of the $b$ component is shorter than that of the $a$ component as a consequence of increased transverse electron relaxation arising from close proximity of the $^1$H-methyl groups of Leu35 to the unpaired electron in the $b$ ensemble of the Q39C-R1 spin label (Fig. 2A).

The ratio of the integrated intensity of the $b$ to $a$ components of the $P(r)$ distribution, $P(b)/P(a)$, for [Leu-$\text{CH}_3$/2H]-AviTag-Protein A (Q39C-R1/K88C-R1) shown in Fig. 2D, is given by the sum of three exponentials:

$$P(b)/P(a) = p_b/(1 - p_b) \exp\left[-T(r_{m}^b - r_{m}^a)/2\right]$$

where $p_b$ is the occupancy of the Q39C-R1 nitroxide in the $b$ state, and $r_{m}^b (= 1/T_{m}^b)$ and $r_{m}^a (= 1/T_{m}^a)$ are the phase-memory relaxation rates for Q39C-R1 in the $b$ and $a$ states, respectively. (Note that when taking the ratio of the integrated intensities of the two peaks in the $P(r)$ distribution, the contribution to phase-memory relaxation from the second spin label at K88C-R1 is cancelled out). The signal intensity, $S(T)$ of the Hahn spin echo curve for [Leu-$\text{CH}_3$/2H]-AviTag-Protein A (Q39C-R1) (Fig. 2E) is given by the sum of three exponentials:

$$S(T) = \lambda_1 \exp(-R_{\text{ESEEM}} T) + \lambda_2[(1-p_b)\exp(-T_{m}^b) + p_b \exp(-T_{m}^a)]$$

where $\lambda_1$ and $\lambda_2$ are scale factors and $R_{\text{ESEEM}}$ is the apparent relaxation rate to account for the very fast initial decay of the spin echo curve due to electron spin echo envelope modulation (ESEEM). Simultaneous fitting of the data in Fig. 2D and E using eqn (1) and (2), yields values of $\sim 23$ and $14$ µs for $T_{m}^a$ and $T_{m}^b$, respectively with a $b$ state occupancy of $\sim 66\%$. The optimized values of the relaxation rates and occupancy of the $b$ state are quantitatively the same (within experimental error) for the $P(r)$ distributions obtained using all five methods of processing the DEER data to generate the $P(r)$ distributions, and are summarized in Table 1.

For [Ile-$\text{CH}_3$/2H] AviTag-Protein A (Fig. 3A), on the other hand, no difference in apparent $T_m$ is observed between singly spin labeled (Q39C-R1 or K88C-R1) or doubly spin labeled (Q39C-R1/K88C-R1) samples (Fig. 3B), and no change in the ratio of peak intensities of the $a$ and $b$ components is observed in the DEER derived $P(r)$ distributions obtained with evolution times $T \geq 2\tau_2$ ranging from 10 to 60 µs (Fig. 3C and D). This is as expected, as the two Ile $\text{CH}_3$ methyl groups are not in close proximity to either the Q39C-R1 or K88C-R1 spin label (Fig. 3A and S2, ESI†), and provides a control for the results obtained with the [Leu-$\text{CH}_3$/2H] labeling.

In summary, we have shown that the introduction of a protonated methyl group(s) in an otherwise fully deuterated background, located in close proximity (less than 8 Å) to a nitroxide spin label can be used as a $T_m$ filter in DEER spectroscopy. The example presented here of AviTag-Protein A provides proof of principle as the presence of a bimodal $P(r)$ distribution is due to two distinctly different regions of conformational space sampled by one of the spin labels (specifically Q39C-R1). However, one can readily envision applications involving complexes between multimeric proteins. For example, in a complex between monomeric and homodimeric proteins the presence of three spin labels cannot be avoided if one wishes to probe distances between the two partner proteins in the complex. When the methyl group(s) is close to the nitroxide spin label in each subunit of the dimer, the relative contribution of the intersubunit distance can be selectively reduced by $T_m$ filtering in the DEER-derived $P(r)$ distribution relative to those of the intermolecular distances between monomer and dimer; conversely, a methyl group close to the nitroxide spin label on the monomer can be used to selectively reduce the contribution of the distances between monomer and dimer relative to the intersubunit distance within the dimer. Similar applications can be envisaged in the context of assigning interactions in multimeric proteins spin-labeled at three sites simultaneously.

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Notes and references


Conflicts of interest

There are no conflicts to declare.